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Immunotherapy of Experimental Malignant Tumors through Activation of Macrophages

—— A Short Review ——

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INTRODUCTION

Cells of a mononuclear phagocyte system, especially monocytes/macrophages, appear to play a central role in cell-mediated immunity. They have an effector non-specific cell function and they are necessary for the induction and regulation of the immune response. Mononuclear phagocytes can recognize, phagocytes, and eliminate foreign particles and cells, senescent cells, and cellular debris, and can also mediate direct non-specific cytotoxicity against pathologically altered cells, such as sickled red blood cells, herpesvirus-infected cells, and tumor cells (for excellent review see Ref. (26)). The uptake, processing, and presentation of antigens to appropriate T and B cells leads to the activation of immune response and to the development of characteristic cellular and/or humoral responses. Macrophages are capable of presenting antigenic determinants in highly immunogenic forms and can alter a tolerogenic antigen into an immunogenic (81). This type of cell is capable of destroying target cells in the presence of antibody which may either be attached directly to the target cell (antibody-dependent cellular cytotoxicity, ADCC) or the macrophage may be precoated (armed) with the cytophilic antibody against the target cell (22, 40). The secretion of various biologically active materials with different functional actions is far more characteristic for macrophages than previously it has been presumed. Except for enzymes capable of affecting extracellular proteins and products involved in defence processes (1, 4, 42, 47, 60), they can release many factors capable of modulation of the function of the surrounding cells. *In vitro,* macrophages can be stimulated to secrete substances that promote the growth of fibroblasts, endothelial cells, and smooth muscle cells (61). A macrophage-derived growth factor stimulates DNA synthesis and proliferation of variety of non-lymphoid mesenchymal cell types, including vascular smooth muscle, vascular endothelial cells, and fibroblasts; tumor-associated macrophages are angiogenic *in vivo* (82). A human granulocyte-macrophage colony-stimulating

factor is a neutrophil-activating factor (106). Human macrophages can synthesize and secrete apolipoprotein E and lipoprotein lipase which may play an important role in the plasma lipoprotein metabolism (105). Human monocytes cultured *in vitro* can also release cytostatic protein factors (49, 69).

It has been agreed that one of the major mechanisms by which tumor immunotherapy exerts its effect is through the activation of macrophage tumoricidal activity (55). Studies from several laboratories revealed that macrophages activated by free or liposome-encapsulated immunomodulators acquire anti-tumor activity (7, 26, 30, 76, 80). In this short review a new line of approach to the immunotherapy of tumors through the activation of tumoricidal macrophages by liposome-encapsulated activators is described.

Key words: Tumor immunotherapy, Activated macrophages, Tumoricidal macrophages, Liposome-encapsulated activators

TUMORICIDAL ACTIVITY OF ACTIVATED MACROPHAGES

Macrophage populations can be activated to express bactericidal and tumoricidal activities. Originally the term "macrophage activation" was used in the early 1960's to determine the enhancement of antimicrobial mechanisms *in vivo* (63). Now this term is used to record the various morphological, functional, and biochemical alterations of macrophages that are different from the properties of resident unstimulated macrophages (93). The macrophage stimulation is often described as a multistep process in which an immature monocyte differentiates into a macrophage that is microbicidal or tumoricidal (11). A single population of cells may not express both bactericidal and tumoricidal activities. Freshly obtained human monocytes are bactericidal but cultured *in vitro* for 5-7 days they lose their activity, even though they acquire many phenotypical characteristics of mature macrophages, such as increased cell size, adherence, spreading, phagocytosis, and protein synthesis (15). It has been found that elicited by an acute inflammatory stimulus (proteose peptone) peritoneal macrophages are highly bactericidal but weakly tumoricidal, whereas macrophages elicited by a chronic infection (BCG) are highly tumoricidal and either show no effect on microorganisms or are at best bacteriostatic (11).

Histopathologic studies revealed an accumulation of blood mononuclear cells within tumor tissues (8, 110). The infiltration of mononuclear cells into human and rodent tumors is a commonly observed phenomenon. A significant component of the mononuclear cell infiltrates of human and rodent tumors are macrophages (43, 101). Tumor-associated macrophages were found within different human tumors

including the lung, stomach, rectum, colon, and the breast (9, 99, 100, 103, 110), the thyroid and seminoma (43), soft tissues and others (104, 107). It has been shown that the macrophage number varies from one tumor to another and no univocal correlation between the macrophage content of tumors and their metastatic capability or immunogenicity was found (101). Recent studies revealed that macrophages represent a functionally heterogeneous cell population with both inhibitory and stimulatory activities in the immune response (44, 46).

In the immunobiology of cancer a highly interesting problem is the ability of macrophages to be directly cytotoxic to tumor cells without having any killing effect on normal cells (4). The tumoricidal activity of activated macrophages is immunologically non-specific (independent of transplantation antigens, species-specific antigens, cell cycle time, various phenotypes associated with transformation) and requires cell-to-cell contact (26, 30, 83). However, it has been also suggested (46) that the mononuclear cell infiltrates from immune rats were capable of recognizing gliosarcoma cells specifically. The mechanism by which macrophages distinguish tumor from normal cells is not fully understood. However, recent studies (26) suggest that a natural phospholipid constituent of biological membranes, phosphatidylserine, can play a major role in the process of recognition of pathological cells, including tumor cells, by macrophages.

Non-cytotoxic macrophages can be rendered tumoricidal by the interaction *in vitro* or *in vivo* with diverse natural and synthetic agents. Some of such agents are listed below:

lymphokines with MAF (macrophage activating factor) activity (17, 28, 29, 37, 39, 45, 54, 77, 78, 86, 89, 102),
interferons (39, 51),
BCG (3, 62, 70, 111),
lipopolysaccharides (10, 19, 93, 98),
monosaccharide precursors of E. coli (68).
lectins (56, 66, 72),
muramyl dipeptide (MDP) and derivatives (2, 6, 7, 16, 30, 48, 58, 64),
lysophosphatidylcholine (67),
cross-linked dextran (5),
polyadenylic-polyuridylic acid (57),
cytosine arabinoside (76),
synthetic acyltripeptide (97).

Muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine, MDP) is a minimal structure of mycobacterial cell wall peptidoglycan, which exhibits adjuvant activity and can replace whole mycobacteria in Freund's complete adjuvant (21). It has been shown that 9 of 15 synthetic MDP analogs enhance antibody production

(12). A good correlation between mitogenicity and the adjuvant action of 9 of 15 compounds was observed *in vitro* ; only the adjuvant active molecules stimulate the incorporation of ^{3}H-thymidine by mouse splenic cells (16). MDP enhances non-specific resistance to some infections (13) and also exerts tumor-inhibitory effects (59). It can potentiate a therapeutic activity of several antitumor agents acting synergistically with endotoxins, such as LPS (lipopolysaccharide), ConA (Concanavalin A), and poly A : poly U (polyadenylic-polyuridylic acid), and it leads to the induction of necrosis and regression of experimental and human tumors (5, 57)

Most of these actions of MDP are mediated by the macrophage(30, 58, 64, 65). Activated syngeneic macrophages injected i. v. reduce the formation of pulmonary mouse melanoma colonies (23). The injection of non-specifically cytotoxic BCG-activated macrophages prevents the formation of spontaneous mouse fibrosarcoma metastases without significant changes in the primary tumor dimensions, proportions of necrosis, and vascularization (62). *In vitro*, all tumor cells, both from primary tumors and metastases, are susceptible to the destruction by tumoricidal macrophages (29, 34, 101). The mere presence of macrophages within tumors is not sufficient to cause tumor regression because tumors exert a suppressive effect on macrophage functions (53, 71). Both the accumulation of macrophages at inflammatory sites *in vivo* and macrophage chemotactic responsiveness *in vitro* are suppressed by tumor-derived factors (73, 90). It has been demonstrated that a low molecular factor extracted from different murine tumors is able to inhibit the LPS-induced tumoricidal activity of mouse peritoneal macrophages (90). On the other hand, it is well established that monocytosis commonly occurs in cancer patients (20, 90). DeMulder *et al.* in their studies (18) found in patients with solid tumors or lymphomatous malignancies that the mean monocyte-mediated ADCC was significantly increased as compared with normal controls. This enhancement may be due to an increased expression of monocyte receptors for Fc fragments of immunoglobulins after activation (38). Thus, Fc receptor-positive (FcR^{+}) monocytes possess ADCC properties (18, 72). Other studies have established that FcR^{+} macrophages suppress lectin-induced lymphoproliferation *in vitro* and exhibit a strong cytostatic activity against tumor cells (108, 109). Tumor cell lines were more efficiently inhibited by FcR^{+} than FcR^{-} (Fc receptor-negative) macrophages ; FcR^{+} macrophages showed the cytostatic effect to L 1210 cells twice as great as that of FcR^{-} (109). FcR^{-} macrophages which represent about 15% or less of macrophages are responsible for the stimulation of both allo- and autogenic mixed lymphocyte reactions and additionally for the presentation of antigen (109).

Free lymphokines with MAF activity and free MDP exert their pharmacological effect by binding to a fucoglycolipid receptor on the monocyte/macrophage surface

membrane (85–87). Only a small fraction of macrophages can respond to free lymphokines (83, 85, 86). Studies on the early events of activation of tumoricidal macrophages revealed total inhibitory effects of D-mannose, α-D-mannosidase, and Fc fragment of immunoglobulin on *in vitro* macrophage activation with MAF (102). Preincubation of macrophages with the antiserum to FcIgG impairs the ability of macrophages to be activated with MAF, although this lymphokine can be adsorbed by these cells, suggesting that the binding of FcIgG to Fc receptor switches on an intracellular signal which in turn switches off the MAF-mediated macrophage activation process (102). The mechanisms responsible for the inability of most macrophages to respond to free MAF, and the induction, nature, and decay of tumoricidal activity in activated macrophages are not yet fully understood.

ACTIVATION OF MACROPHAGES BY LIPOSOME-ENCAPSULATED ACTIVATORS

Fidler, Poste, and co-workers from the US' NCI-Frederick Cancer Research Facility and Smith Kline and French Laboratories have paid special attention over the last few years to the induction of tumoricidal activity in macrophages by liposome containing lymphokines (24, 87) or MDP (25, 94). Systemic administration of the lymphokine-containing liposomes leads to the activation of the tumoricidal properties of mouse macrophages *in vivo* and to the eradication of established pulmonary metastases in 73% of mice (24). Similar results were obtained with MDP (27, 35, 92). The effectiveness of MDP injected i. v. in free form is limited by its rapid clearance; at 2 min after injection less than 20% of the dose remains in the bloodstream of a mouse (2) and most of the injected MDP is excreted in urine during the first 2 h (75). MDP entrapped in liposomes has been found to be far more efficient in increasing tumoricidal activity of macrophages than free MDP (94, 96). Human monocytes are stimulated to the cytotoxic activity against allogenic tumor cells *in vitro* by 800–1000 times less liposome-encapsulated MAF than free MAF (52). In addition to the lower doses required for equivalent activation it has been shown in mouse models that the liposome-encapsulated MAF also activates macrophages unresponsive to free MAF by reason of impairment or lack of surface receptors for MAF (31, 85). The activation of macrophages by the liposome-encapsulated MAF requires only that the liposomal vehicle should be internalized by endocytosis (31).

Application of liposomes as carrier vehicles to deliver activators to macrophages in situ was a great methodical step ahead, especially in the context of the therapy of disseminated diseases. The above mentioned investigators have found the optimal type of liposome for the delivery of the macrophage-activating agents to reticuloendothelial cells (32, 84, 92). On the basis of a comparison of the

liposomes studied it has been established that large (1-2 μm in diameter) multilamellar liposomes prepared from phosphatidylcholine (neutral) and phosphatidylserine (negative) in the 7 : 3 molar ratio represent the optimal type (33, 88).

There are the following ways of the liposome-cell interaction : stable adsorption, endocytosis, fusion, and lipid transfer (33, 74). The majority of phosphatidylcholine-phosphatidylserine liposomes are uptaken by endocytosis (31, 88) in organs with a high reticuloendothelial activity, such as the spleen and lymph nodes, and are accumulated in a low grade in other organs, such as the lung or kidney (32).

Fusion of these liposomes with the macrophage plasma membrane with a simultaneous release of the encapsulated material directly into the cytoplasm is not quantitatively significant (88).

Toxicity studies of i. v. injections of large doses of liposome-encapsulated lymphokines did not reveal any detectable toxicity or tissue changes (41). The phospholipid products of the intracellular degradation of liposomes, at least those of the composition used in the referred studies, can be either exhausted in the process of cellular biosynthesis or eliminated from the cell by excretion, or else they can disappear in both ways (36).

Negatively charged liposomes injected i. v. avoid liver and spleen entrapment and penetrate through cell layers into the lung alveolus to be engulfed there by alveolar macrophages (91). However, the best documented explanation of this phenomenon has been proposed by Poste *et al.* (84). Their results indicate that the limited transcapillary transport of liposomes occurs in open sinusoidal capillaries (liver) but does not occur in organs with continuous capillaries (lung) even in tumors vascularized by capillaries with structural defects. Liposomes in the lung capillaries are engulfed by circulating blood monocytes which subsequently migrate to the extravascular alveolar tissue, turning into alveolar macropages (84).

Fidler *et al.* (27) provided evidence that the eradication of established pulmonary and lymph node metastases in mice following systemic administration of liposomes containing macrophage activators is mediated by tumoricidal macrophages. Systemic administration of the agents which impair macrophage function, such as carrageenan, silica, and hyperchlorinated drinking water, is associated with an increased incidence of metastases (27, 101). It has been shown that a lymphokine with MAF activity, which is heat stable (2-5 min at 100℃), can activate normal non-cytotoxic macrophages to a tumoricidal state (52, 86). According to Kleinerman *et al.* (51), the activation is distinctly different from augmentation which leads to the enhancement of cytotoxic properties in macrophages showing spontaneous cytotoxicity. Such spontaneous cytotoxicity has been found in human alveolar macrophages (95). Similarly, human monocytes freshly

isolated from normal donors are highly tumoricidal without further stimulation, but following treatment with liposomes containing MDP or its lipophilic analog muramyl tripeptide (MTP) monocytes remain tumoricidal to allogenic melanoma cells for up to 5 days (50, 96). Previous studies demonstrated defective monocyte and macrophage cytotoxic function in both animals and patients with tumors (14, 53, 71, 79). In the opinion of Sone *et al.* (96) spontaneously tumoricidal monocytes participate in the control of metastatic spread of tumor cells in the peripheral circulation. This is also in agreement with an earlier observation of experimental tumor models (62) in which the BCG-activated macrophages can reduce the entry of tumor cells into vessels inside primary tumors.

The success of therapy with the liposome-encapsulated macrophage activators is limited when the tumor load is large (24, 30). It has been shown that macroscopic pulmonary metastases of mouse B 16 melanoma do not respond to liposomal MDP treatment (48, 80). However, it seems that the potential application of this therapy is not applicable to the treatment of large solid tumors (30), which are surgically resected routinely, but rather the destruction of micrometastases, especially pulmonary and lymph node metastases. According to Sone *et al.* (96), the liposome-encapsulated MDP or MTP should be tested for its enhancing ability of the tumoricidal activity of human monocytes in situ, because this course of action is potentially useful in clinical therapy.

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